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Delayed Leaf Senescence in Tobacco Plants Transformed with *tmr*, a Gene for Cytokinin Production in *Agrobacterium*

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The aim of this study was to investigate whether enhanced levels of endogenous cytokinins could influence plant development, particularly leaf senescence. Tobacco plants were transformed with the *Agrobacterium tumefaciens* gene *tmr*, under the control of the soybean heat shock promoter HS6871. This gene encodes the enzyme *iso*-pentenyl transferase, which catalyzes the initial step in cytokinin biosynthesis. After heat shock, the cytokinin level increased greatly and the level of *tmr* mRNA, undetectable at 20°C, rose and remained high for up to 8 hours. The levels of cytokinin and *tmr* mRNA were substantially lower by 24 hours. Transformed plants grown at 20°C were shorter, had larger side shoots, and remained green for longer than untransformed plants. The differences were more pronounced after several heat shocks of whole plants or defined areas of leaves. Our results demonstrated that plant morphology and leaf senescence can be manipulated by changing the endogenous level of cytokinins.

INTRODUCTION

Plant growth and development are thought to be regulated by the interaction of a range of phytohormones, of which cytokinins are just one type. The term cytokinin was coined after the discovery of a substance that stimulated cell division, or cytokinesis, in cultured tobacco cells. Subsequently, it has been found that external application of cytokinins produces a variety of additional effects on plants, including the release of lateral buds from apical dominance (Sachs and Thimann, 1964), alteration of source/sink relationships (Leopold and Kawase, 1964), counteraction of the effects of heat stress (Caers et al., 1985), stimulation of chlorophyll synthesis (Arnold and Fletcher, 1986), and delaying of senescence (Richmond and Lang, 1957).

Senescence is the sequence of biochemical and physiological events comprising the final phase in development, which culminates in cellular breakdown and death. Medawar (1957) provided a convenient distinction between senescence and aging by defining the latter as referring to all those changes that occur with time, without reference to death as a consequence. The first visible sign of leaf senescence is yellowing, which is due to the preferential degradation of chlorophylls in comparison with xanthophylls and carotenoids (Gut et al., 1987). There is a general increase in the degradation of proteins and nucleic acids during senescence, although some new synthesis of these is necessary (Thomas and Stoddart, 1980).

Despite the fact that senescence results in the destruction of the cell's contents, it is thought that the changes taking place form a highly coordinated, genetically programmed sequence, mediated by specific chemical signals (Roberts and Hooley, 1988). Factors influencing the initiation of senescence include new gene expression; competition for space, light, nutrients, and growth regulators; and environmental stresses, such as extremes of temperature, water and mineral availability, and invasion by pathogens (Thomas and Stoddart, 1980).

At present, there are two types of evidence for the role of cytokinins in the control of senescence. One is that external application of cytokinins to detached leaves delays their senescence (Richmond and Lang, 1957) and may cause regreening of yellow leaves in some species (Dyer and Osborne, 1971). The other evidence is purely correlative and may be summarized as follows. Root exudates and rooting extend leaf longevity, and exudates have been found to contain cytokinins (Van Staden et al., 1988). Disbudding of plants increases cytokinin export by the roots and delays senescence of the leaves (Colbert and Beever, 1981). The level of cytokinins exported by the

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roots decreases sharply when plants reach maturity and begin to senesce (Sitton et al., 1967). "Green islands" around sites of pathogen infection contain enhanced levels of cytokinins (Király et al., 1967).

There are problems associated with the interpretation of both types of evidence. For instance, if the cytokinin is applied externally, its efficiency of uptake and transport must be considered, and these factors are incompletely understood. In correlative studies, it cannot be proved that a change in the level of cytokinins causes the observed effect. Furthermore, the levels of cytokinins were measured using bioassays, in which the cytokinin activity may differ from that in delaying senescence. In this paper, we describe a system for increasing the endogenous level of cytokinins by the controlled induction of a key enzyme in cytokinin biosynthesis, which avoids the problems of external application (see also Medford et al., 1989; Schmülling et al., 1989). In addition, we have measured the level of cytokinins using HPLC followed by radioimmunoassays, which provide more precise qualitative and quantitative data than bioassays.

There are three main families of naturally occurring cytokinins: the zeatin, *iso*-pentenyl adenine, and dihydrozeatin types. They are all N^6-substituted adenine derivatives and differ only in the composition of the side chain, which confers their biological activity. Interconversions within and between families of cytokinins may occur, e.g., ribotide ⇌ riboside ⇌ free base within each family and *iso*-pentenyl adenine types → zeatin types → dihydrozeatin types between families (McGaw, 1988). Such modifications may provide regulation of the level of biologically active cytokinins because the free base may be the most active form of cytokinin in each family (Laloue and Pethe, 1982). Inactivation of cytokinins is achieved by removal of the side chain using the enzyme cytokinin oxidase.

The initial step in cytokinin biosynthesis involves the addition of an *iso*-pentenyl side chain to 5′-AMP in the following reaction (Chen and Melitz, 1979):

iso-pentenyl pyrophosphate + 5′-AMP
↓
iso-pentenyl adenine ribotide + pyrophosphate

In *Agrobacterium tumefaciens*, this reaction is catalyzed by an *iso*-pentenyl transferase that is encoded by the gene *tmr* (Barry et al., 1984), and it is thought to be the rate-limiting step in cytokinin synthesis (Akiyoshi et al., 1984).

We have transformed tobacco plants with the *Agrobacterium* gene *tmr*, under the control of the soybean heat shock promoter HS6871 (Schöffl et al., 1984). The transformed plants have been used to investigate the effect of increasing the endogenous cytokinin level of the whole or part of the plant by heat shock at a specific developmental stage.

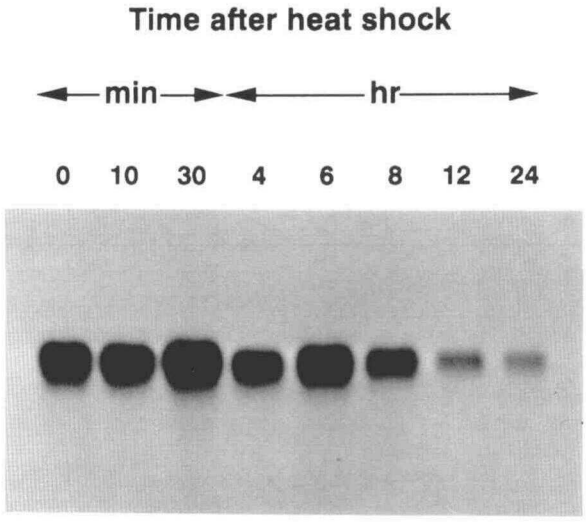

Figure 1. Changes in *tmr* mRNA Levels in Leaves of Transformed Tobacco Plants after Heat Shock.

Whole transformed plants were heat shocked at 42°C for 2 hr, and then individual leaves were removed for poly(A)$^+$ RNA extraction at the specified times after the end of the heat treatment. Equal amounts of poly(A)$^+$ RNA from each leaf were electrophoresed in a formaldehyde-agarose gel and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled *tmr* gene as the probe and washed, and the remaining signal was visualized by autoradiography.

RESULTS

Construction of Transgenic Plants Containing an Inducible *iso*-Pentenyl Transferase Gene

A 405-bp fragment of the soybean heat shock promoter HS6871 was joined to a 1.4-kb fragment of the *tmr* gene from the pTi T37 T-DNA of *Agrobacterium*. The construct was transferred to the binary vector pBin19, mobilized into *Agrobacterium*, and used to transform tobacco leaf discs. Nine independent primary transformants were regenerated and shown to contain the pHS6871::*tmr* construct by DNA gel blot analysis. One of these primary transformants, which contained three copies of the insert, was used for all subsequent experiments.

Expression of the *tmr* Gene after Heat Shock

Mature soil-grown transformed tobacco plants were heat shocked at 42°C for 2 hr, and immediately afterward the *tmr* mRNA was readily detectable by RNA gel blot analysis. The primary transformant with the highest level of *tmr* mRNA after heat shock was used for all subsequent experiments. There was no detectable cross-hybridization

Table 1. Measurements of Cytokinin Levels 4 hr after Heat Shock

Type of Tissue	pmol ZR equivalents/g fresh wt (SE)			pmol IPR equivalents/g fresh wt (SE)			pmol DZR equivalents/g fresh wt (SE)		
	ZRP	Z	ZR	IPRP	IP	IPR	DZRP	DZ	DZR
Transformed heat-shocked area	54.6 (9.5)	120.6 (8.6)	35.1 (6.1)	8.3 (2.4)	42.7 (10.6)	2.6 (0.8)	11.1 (1.8)	17.1 (1.0)	3.0 (0.4)
Transformed rest of leaf	7.4 (4.0)	9.0 (3.9)	4.4 (2.6)	1.3 (0.5)	5.5 (1.3)	0.8 (0.4)	1.8 (0.5)	2.1 (0.8)	0.4 (0.2)
Untransformed heat-shocked area	1.6 (0.5)	0.8 (0.4)	0.6 (0.3)	1.5 (0.2)	0.2 (0.2)	0.3 (0.3)	1.9 (0.4)	1.1 (0.1)	0.9 (0.3)
Untransformed rest of leaf	0.4 (0.1)	0.1 (0.1)	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)	ND[a]	0.2 (0.1)	0.5 (0.2)	0.5 (0.4)

A rectangular area of leaf was heat shocked at 42°C for 2 hr. After this treatment, the leaf was left on the plant at 20°C for 4 hr before harvesting of the heat-shocked area separately from the rest of the leaf. Cytokinins were extracted separately from the two parts of the leaf, fractionated by HPLC, and quantified using radioimmunoassays for zeatin-, *iso*-pentenyl adenine-, and dihydrozeatin-type cytokinins. Each value represents the mean and SE of three measurements made on different leaves. The abbreviations used are Z, zeatin; IP, *iso*-pentenyl adenine; DZ, dihydrozeatin, with the suffixes RP, ribotide; R, riboside.

[a] ND, not detectable.

between the *tmr* probe and any mRNA in untransformed plants or in transformed non-heat-shocked plants, even after exposure of autoradiograms for a long period (data not shown). The level of *tmr* mRNA in transformed plants remained at a high level for up to 8 hr after heat shock, but by 24 hr the level had decreased considerably, as shown in Figure 1.

Production of Cytokinins after Heat Shock

Zeatin, *iso*-pentenyl adenine, and dihydrozeatin types of cytokinin were measured in yellowing leaves of transformed and untransformed tobacco plants. A rectangular area of leaf was heat shocked at 42°C for 2 hr, and then the leaf was left on the plant for either 4 or 24 hr before harvesting of the heat-shocked tissue separately from the rest of the leaf. The cytokinins were extracted in 80% methanol, fractionated by HPLC, and quantified using radioimmunoassays. Separate measurements were made from three leaves of both transformed and untransformed plants at each of the two time points after heat shock.

A massive increase in cytokinins was detected 4 hr after heat shock of transformed leaves, with zeatin-type cytokinins being the most abundant of the three families measured, as shown in Table 1 and Figure 2A. The level of zeatin was 17-fold higher in the heat-shocked area than in the rest of the leaf, zeatin ribotide was sevenfold higher, and zeatin riboside eightfold higher. The tissue surrounding the heat-shocked zone had a higher level of cytokinins in transformed than untransformed leaves. Figure 2B illustrates that in untransformed leaves the level of cytokinins was found to be consistently slightly higher in the heat-shocked area than in the rest of the leaf.

The level of cytokinins had decreased substantially by 24 hr after heat shock of transformed leaves, as shown in Figure 2C. In the non-heat-shocked part of the transformed

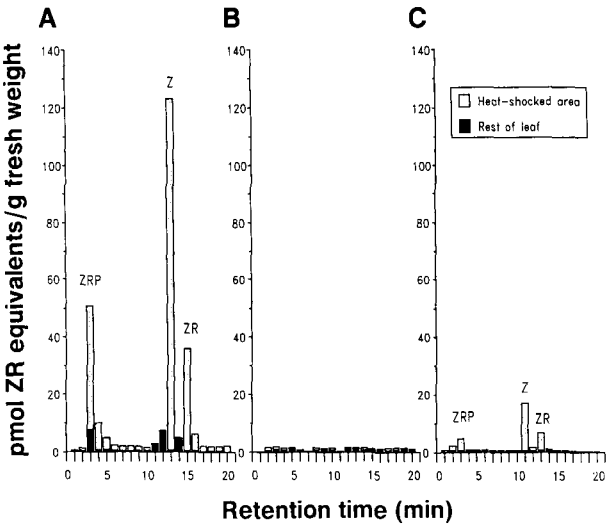

Figure 2. Zeatin-Type Cytokinin Content in Transformed and Untransformed Tobacco Leaves.

A rectangular area of a yellowing tobacco leaf was heat shocked at 42°C for 2 hr, and then the leaf was left on the plant at 20°C for either 4 or 24 hr. After this time, the heat-shocked area was harvested separately from the rest of the leaf. Cytokinins were extracted separately from the two parts of the leaf, separated by HPLC, and quantified by radioimmunoassay. The values plotted represent the mean of three sets of measurements made on separate leaves.

Abbreviations are as given in Table 1.

(A) Zeatin-type cytokinin content 4 hr after heat shock of leaves of plants transformed with the pHS6871::*tmr* construct.

(B) Zeatin-type cytokinin content 4 hr after heat shock of leaves of untransformed plants.

(C) Zeatin-type cytokinin content 24 hr after heat shock of leaves of plants transformed with the pHS6871::*tmr* construct.

Table 2. Measurements of Cytokinin Levels 24 hr after Heat Shock

	pmol ZR equivalents/g fresh wt (SE)			pmol IPR equivalents/g fresh wt (SE)			pmol DZR equivalents/g fresh wt (SE)		
Type of Tissue	ZRP	Z	ZR	IPRP	IP	IPR	DZRP	DZ	DZR
Transformed heat-shocked area	4.9 (3.0)	16.2 (10.6)	5.9 (4.6)	0.3 (0.2)	0.6 (0.3)	0.5 (0.2)	1.6 (0.1)	0.4 (0.4)	0.7 (0.3)
Transformed rest of leaf	0.4 (0.1)	0.4 (0.1)	0.5 (0.2)	0.1 (0.0)	0.4 (0.2)	0.2 (0.1)	0.4 (0.2)	0.7 (0.2)	1.5 (0.6)
Untransformed heat-shocked area	0.5 (0.2)	0.5 (0.1)	0.7 (0.1)	0.2 (0.2)	0.5 (0.2)	0.2 (0.1)	0.4 (0.2)	0.5 (0.1)	0.9 (0.3)
Untransformed rest of leaf	0.5 (0.2)	0.4 (0.1)	0.3 (0.1)	0.2 (0.2)	0.1 (0.1)	0.1 (0.1)	0.8 (0.2)	0.5 (0.2)	0.4 (0.1)

The methods used were identical to those described in Table 1, except that the leaves were left on the plants for 24 hr, rather than 4 hr, after the heat treatment. Each value represents the mean and SE of three measurements made on separate leaves. Abbreviations are as given in Table 1.

leaf, the level of cytokinins was similar to that in the equivalent part of untransformed leaves by 24 hr after heat shock, as detailed in Table 2. This represents a considerable decrease in the level of cytokinins in the area surrounding the heat-shocked part of the leaf between 4 and 24 hr after heat shock.

Trends in cytokinin levels in different samples were reproducible, although there was some variation in the measured values. The position of the leaf on the stem was found to be an important factor; therefore, efforts were made to choose leaves at a similar height on the stem for each set of measurements.

To establish the efficiency of extraction, fractionation, and quantification of cytokinins, duplicate samples of the non-heat-shocked areas of transformed and untransformed leaves 4 hr after heat shock were prepared and 3 pmol of each of the 12 quantifiable cytokinins was added to one of each pair of samples before extraction. This amount was chosen because it was within the range of cytokinin levels measured in the tissues studied. Comparison between the cytokinin levels of the paired samples recorded in Table 3 revealed a high percentage recovery of the 12 cytokinins for both types of tissue. This suggested that the procedure employed was capable of measuring accurately the amount of each cytokinin, if present. However, the recovery of the ribotide form of each family of cytokinins was consistently lower than that of the other types of cytokinin. It is likely that phosphatase activity in the tissue may have converted the ribotide to the corresponding riboside. This would explain why the recovery of riboside was sometimes more than 100%. In addition, each fraction was measured in all three radioimmunoassays. Because every peak of cytokinin was accompanied by low values in the other two assays, we believe that there is no evidence that the results presented here were due to nonspecific interference.

An extraction medium has been developed by Bieleski (1964) to minimize phosphatase activity, but this was not used because Turnbull and Hanke (1985) found that it resulted in severe interference in the radioimmunoassay. Thus, the values for amounts of cytokinin ribotides are probably underestimates. In addition, because the antibodies used in the radioimmunoassays were raised to the riboside in each case, only the riboside values can be regarded as absolute. The amounts measured of the ribotide, 9-glucoside, and free base depend on the cross-reactivity of the antibody to the compound in question. Because of the polyclonal nature of the antiserum, its cross-reactivity to a particular cytokinin differs depending on the amount present. Therefore, levels of all cytokinins within one family may be expressed only as amounts of cytokinin riboside equivalents. This is in contrast to measurements made by gas chromatography-mass spectrometry, in which absolute values for individual cytokinins may be obtained, as in Medford et al. (1989).

Effects of Increased Cytokinins on the Morphology and Rate of Senescence of Plants

It is likely that there is some extra cytokinin production in transformed tobacco plants grown at 20°C compared with untransformed plants. Figure 3A shows that transformed plants tend to be shorter, with larger side shoots, and that individual leaves stay green for longer. Because no *tmr* mRNA could be detected, the level of expression was presumably very low.

A single heat shock of an entire transformed plant did not appear to cause any additional change in morphology in comparison with an untransformed plant. Therefore, it was decided to give plants a weekly heat shock at 42°C for 2 hr. Plants grown in tissue culture were selected for this treatment because of their ability to withstand repeated heat shocks better than soil-grown plants. This was probably because the tissue culture containers provided protection from desiccation. After 12 weeks, the plants were as shown in Figure 3B. The transformed heat-shocked plant exhibited an abundance of tiny shoots at the apex and a release of lateral buds, which was not

Table 3. Recovery of Zeatin-, *iso*-Pentenyl Adenine-, and Dihydrozeatin-Type Cytokinins from Transformed and Untransformed Leaves

Type of Tissue	Type of Cytokinin	Recovery of Individual Cytokinins (%)	Total Recovery of Added Cytokinins (%)[a]
Transformed non-heat-shocked area of heat-shocked leaf	ZRP	50	91
	Z9G	106	
	Z	99	
	ZR	101	
	IPRP	68	98
	IP9G	118	
	IP	73	
	IPR	118	
	DZRP	76	110
	DZ9G	123	
	DZ	113	
	DZR	129	
Untransformed non-heat-shocked area of heat-shocked leaf	ZRP	47	88
	Z9G	109	
	Z	92	
	ZR	97	
	IPRP	75	90
	IP9G	88	
	IP	88	
	IPR	99	
	DZRP	51	81
	DZ9G	102	
	DZ	76	
	DZR	97	

Duplicate samples were taken of the area of leaf surrounding the heat-shocked part from transformed and untransformed leaves. Three picomoles of each of the 12 quantifiable cytokinins was added to one of each pair of samples before extraction of cytokinins. The percentage recovery for each cytokinin was calculated from the difference in the amount measured between the two samples of the same tissue, in comparison with the amount added. Abbreviations are as given in Table 1; 9G, 9-glucoside.

[a] For each type of tissue, the overall percentage recovery within each family of cytokinins was calculated by adding the amount of extra cytokinin measured for each of the four types and dividing by the sum of the amounts added.

found in the untransformed heat-shocked plant. Transformed non-heat-shocked plants consistently remained green the longest.

Further work was carried out using a localized heat shock on part of a single leaf, and the effect on the treated leaf and the rest of the plant was noted. After a single heat shock of a transformed leaf, the heat-shocked area remained slightly greener than the rest of the leaf, and the senescence of leaves higher up the stem was accelerated. This change in source/sink relationships was also seen, but to a lesser extent, after heat shock of a leaf from an untransformed plant. Multiple heat shocks of part of one leaf had a much more dramatic effect, just as was seen for the heat shock of whole plants. Figure 3C illustrates that after four heat shocks at 42°C for 2 hr on the same area of leaf, with 3 to 4 days between treatments, the heat-shocked area of the transformed leaf remained green while the rest of the leaf turned yellow. In contrast, the untransformed leaf died. Caers et al. (1985) reported that externally applied cytokinin could reverse the symptoms of heat shock. Our result suggested that extra cytokinin produced endogenously in the transformed leaf may have the same effect.

DISCUSSION

Transformation of plants with the *Agrobacterium* T-DNA gene *tmr* under the control of a constitutive promoter results in plants of abnormal morphology that produce many shoots but no roots (Schmülling et al., 1989). Regulation of the *tmr* gene can be achieved using an inducible promoter. In this study, we used the heat shock promoter HS6871 from soybean to control the activity of the *tmr* gene. Soybean and tobacco are both dicotyledonous plants, and the soybean promoter has already been shown to be active in transformed tobacco plants (Baumann et al., 1987). The heat shock response of plants is induced by increasing the temperature to about 40°C and consists of the termination of transcription and translation of most genes and the initiation of synthesis of a new set of proteins, known as heat shock proteins (Key et al., 1981).

We have found that the soybean heat shock promoter pHS6871 does function correctly in tobacco plants because transient *tmr* expression occurs upon heat shock. This is demonstrated both at the mRNA level by RNA gel blot analysis (Figure 1) and in the level of cytokinins (Figure 2). *tmr* must produce a functional *iso*-pentenyl transferase because the level of cytokinins increases dramatically after heat shock of transformed plants (Figure 2). Evidence from cytokinin measurements is corroborated by the "shooty" appearance of the transformed tissue-cultured plant after multiple heat shocks (Figure 3B) and the delayed senescence of the heat-shocked part of a transformed leaf (Figure 3C), both effects that may be obtained by external application of cytokinins.

The initial product of the reaction catalyzed by *iso*-pentenyl transferase is *iso*-pentenyl adenine ribotide, but this seems to be converted rapidly into zeatin-type cytokinins in the plant because by 4 hr after heat shock zeatin-type cytokinins predominated (Table 1). The ability of the

Figure 3. Morphology and Degree of Senescence of Transformed and Untransformed Plants and Individual Leaves under Different Temperature Regimes.

(A) Transformed (left) and untransformed (right) plants grown to maturity in John Innes No. 1 compost. The plants were grown in a controlled environment chamber with a 16-hr photoperiod and 90% relative humidity at 20°C.
(B) Tissue-cultured plants with or without 12 weekly heat shocks at 42°C for 2 hr. From left to right: transformed heat-shocked plants, untransformed heat-shocked plants, transformed non-heat-shocked plants, and untransformed non-heat-shocked plants.
(C) Leaves on transformed (left) and untransformed (right) plants after 4 heat shocks at 3- to 4-day intervals of the same area of leaf. Each heat shock was carried out on a rectangular area of leaf at 42°C for 2 hr.

transformed plant to produce a high level of cytokinins by 4 hr after heat shock suggests that the necessary metabolites and enzymes, other than *iso*-pentenyl transferase, are either present in excess or may be produced rapidly in response to the dramatic rise in *iso*-pentenyl adenine-type cytokinins.

The level of cytokinins is substantially lower by 24 hr after heat shock (Table 2), which suggests that there is an efficient pathway for their degradation in the leaf and that *iso*-pentenyl transferase has a high rate of turnover. It is possible that the greatly increased amount of cytokinins produced after heat shock of a transformed leaf may trigger the synthesis of a correspondingly high level of degradative enzymes, such as cytokinin oxidase. Chatfield and Armstrong (1986) have provided some evidence that external application of cytokinins to callus tissue results in increased cytokinin oxidase activity. This might explain why the cytokinin content of the rest of the leaf surrounding the heat-shocked area is also much lower 24 hr after heat shock. Cytokinins diffusing from the heat-shocked area into the rest of the leaf might activate the degradation pathway to such an extent that the balance swings temporarily toward excess breakdown and does not return to equilibrium until after 24 hr.

Some of the extra cytokinin present in the rest of the leaf 4 hr after heat shock may be attributable to diffusion from the heat-shocked area, but probably not all of it because there is evidence for an increased cytokinin content in transformed plants grown at 20°C. These plants are shorter, have larger side shoots, and exhibit delayed senescence in comparison with untransformed plants (Figure 3A). Measurement of cytokinin levels in the leaves of transformed and untransformed plants not subjected to heat shock over any part of the leaf would assist in the interpretation of this result.

At present, the visual evidence from transformed plants grown in soil and tissue culture suggests that some extra cytokinin is being produced at 20°C, implying that the heat shock promoter may be slightly "leaky." This raises the question of whether or not this heat shock promoter is completely inactive at normal growth temperatures. At least some heat shock proteins are thought to have a role in metabolism at normal temperatures as well as on heat shock (Ostermann et al., 1989). The "leakiness" is not detectable at the mRNA level, but a very small amount of message might be sufficient to produce enough enzyme to increase the cytokinin level such that it would affect the morphology of the plant. Alternatively, this phenotype might be due to somaclonal variation during the transformation process causing cytokinin habituation.

In untransformed plants, the level of cytokinins was found consistently to be slightly higher in the heat-shocked area than in the rest of the leaf. Perhaps increased cytokinin production is a natural response to stress. Because of the transient nature of *tmr* expression after heat shock, multiple heat shocks of whole plants in tissue culture (Figure 3B) or the same area of a single leaf (Figure 3C) enhanced greatly the difference between transformed and untransformed plants. The response of a transformed leaf to multiple heat shocks merits further study. Measurements of chlorophyll and photosynthetic rate in the treated area and the rest of the leaf would reveal the extent to which the green appearance correlates with physiological activity.

A similar construct, using the maize *hsp*70 promoter linked to the *tmr* gene, has been transferred to tobacco plants by Medford et al. (1989). They also found that zeatin-type cytokinins were the major forms produced on heat shock and that a lower level of cytokinins accumulated at 20°C. Another similarity between the two investigations is in the difference in appearance between transformed and untransformed plants at the control temperature, the transformed plants being shorter and having larger side shoots than untransformed plants. However, they found no differences in morphology between heat-shocked and non-heat-shocked transformed tobacco plants, which may be because the increase in the cytokinin level is transient. In contrast, we found that after multiple heat shocks there were obvious differences between heat-treated and control transformed plants, both in tissue culture and using individual leaves attached to soil-grown plants. It is possible that this difference between the two studies may be related to the method of heat shock. Medford et al. (1989) raised the temperature gradually, whereas we imposed a sudden heat shock, which may have activated the heat shock promoter more effectively. Alternatively, it may be more difficult to detect a response using whole plants in soil rather than tissue-cultured material or individual plant parts. Treatment of only a part of the plant was found to facilitate detection of the response to heat shock because it allowed for comparisons to be made more easily.

Schmülling et al. (1989) have also made transgenic plants containing the *tmr* gene but under the regulation of the *Drosophila hsp*70 promoter. They obtained similar results to those presented here for the amount of zeatin ribotide in leaves of untransformed plants. However, in contrast to the work of Medford et al. (1989) and that described here, Schmülling et al. (1989) reported that transformed plants were similar in appearance to untransformed plants and that there was no difference in cytokinin levels between the two types of plant at normal growth temperatures. Consequently, it is possible that the *Drosophila hsp*70 promoter may exert tighter regulation at the control temperature than either the maize *hsp*70 or soybean HS6871 promoters. Another possibility is that the transformed plants of Schmülling et al. (1989) contained the *tmr* gene, but that it was switched off at some point during development (Spena and Schell, 1987).

The results described here demonstrate that the endogenous level of cytokinins in plants may be manipulated by transforming them with the *tmr* gene under appropriate

control. We have used this system to provide direct evidence for the role of cytokinins in the control of leaf senescence, for when the endogenous cytokinin level of an area of transformed leaf is increased by heat shock, its senescence is delayed. The effect is similar to that of the external application of cytokinins and green islands around sites of pathogen attack, where cytokinins have been detected (Király et al., 1967). However, the approach presented here avoids the need to consider uptake and transport efficiency or to distinguish between cause and effect. Furthermore, changes in cytokinin levels can affect plant morphology dramatically, confirming that these substances play an important role in plant development.

Thus, if a gene such as *tmr* were introduced into plants under the control of a suitable developmentally regulated promoter so that it was activated at an appropriate stage in the life cycle, it might be possible to influence the timing of senescence. Studies on slow-senescing mutants of several crop plants, including sorghum (Ambler et al., 1987), have already shown the importance of delayed senescence in the increasing of plant yield.

METHODS

Vector Construction

A 405-bp XbaI-XhoI fragment from the soybean heat shock promoter HS6871 (Schöffl et al., 1984) was blunt ended and then inserted into the HincII site of a modified pUC19 plasmid, containing a 260-bp nopaline synthase transcription terminator cloned into the SstI site. The XhoI site of the promoter was generated 4 bp upstream of the transcription start site of the heat shock gene by deletion of a longer fragment, followed by the addition of XhoI linkers. Next, the *tmr* gene (Goldberg et al., 1984) was cloned into the SalI and SmaI sites of the same plasmid as a 1.4-kb XhoI partial-blunted HindIII fragment. This fragment contained 604 bp of the *tmr* gene's 3′-untranslated region. The XhoI-SalI fusion ensured that the coding region of the *tmr* gene was in the correct orientation. Finally, the construct was transferred to pBin19 (Bevan, 1984) as a 2.07-kb HindIII-EcoRI fragment to generate the vector pBI155.2. This was mobilized from *Escherichia coli* HB101 to *Agrobacterium tumefaciens* LBA4404 by triparental mating, with HB101 containing pRK2013 as the helper strain.

Plant Transformation

Transformation of tobacco leaf discs with *Agrobacterium* cells containing pBI155.2 was carried out according to the method of Horsch et al. (1985).

Plant Material

Plants of *Nicotiana tabacum* cv Samsun were grown in Magenta GA-7 pots containing the revised medium of Murashige and Skoog (1962) with an 18-hr photoperiod at 25°C. When soil-grown material was required, plants were transferred to John Innes No. 1 compost and grown in a controlled environment chamber with a 16-hr photoperiod and 90% relative humidity at 20°C.

DNA Gel Blot Analysis

Integration of the construct into the genomic DNA of each transformant was confirmed by DNA gel blot analysis (Southern, 1975, as modified by Sharp et al., 1988). The probe used was the 1.4-kb XhoI-HindIII fragment of the *tmr* gene, which was labeled with α-^{32}P-dCTP by oligolabeling (Feinberg and Vogelstein, 1984).

Methods of Heat Shock

Three different methods of administering the heat shock were used, but in all cases the heat shock treatment was for 2 hr at 42°C. First, mature tobacco plants in soil were placed in a heated cabinet and sprayed with water every 15 min to minimize desiccation. Second, tissue-cultured tobacco plants in Magenta GA-7 pots were put in an incubator. Third, a rectangular area of a single leaf (10 × 4 cm) was treated while still attached to the plant, using a Perspex air-flow chamber clamped onto the leaf. A metal block below the chamber was heated with water circulated from a water bath, while the air within the chamber itself was heated by passing it through a copper coil immersed in the water bath. The temperature of the air surrounding the leaf was monitored using a thermocouple. The first method was used when leaf tissue was required for mRNA extraction, the second when the effect on morphology and rate of senescence was being investigated, and the third when measurements of cytokinin content were being made.

RNA Gel Blot Analysis

Polyadenylated RNA was extracted from leaves using the procedure of Apel and Kloppstech (1978), which included selection by oligo(dT)-cellulose column chromatography. The poly(A)$^+$ RNA was fractionated in 1.5% agarose gels containing formaldehyde and transferred to a nylon membrane. Expression of the *tmr* mRNA was analyzed by hybridization of the ^{32}P-labeled *tmr* gene to the RNA on the membrane. Hybridization was carried out at 42°C for 16 hr in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 0.1 mg/mL salmon sperm DNA (based on Sambrook et al., 1989). The membrane was washed four times in 2 × SSC, 0.1% SDS, and twice in 0.1 × SSC, 0.1% SDS, each wash being for 5 min at room temperature, and the remaining signal was visualized by autoradiography.

Extraction of Cytokinins

A rectangular area of leaf was heat shocked as described above and the leaf left on the plant at 20°C for either 4 or 24 hr before harvesting the heat-shocked area separately from the rest of the leaf. The leaf tissue was frozen in liquid nitrogen and ground to a fine powder with a pestle and mortar. Cytokinins were extracted by the addition of 20 volumes of ice-cold 80% methanol with occasional stirring over 1 to 1.5 hr. After centrifugation at 1180*g*

for 10 min and recovery of the supernatant, the pellet was resuspended in 10 mL of ice-cold 80% methanol and recentrifuged, and the supernatants were combined. A rotary evaporator was used at 35°C under vacuum to reduce the volume of extract to 10 to 15 mL.

Pigments and phenolics were removed from the extract with Polyclar AT using the method of Leitch (1990). A slurry was prepared by equilibrating 0.5 g of Polyclar AT with 15 mL of double-distilled water, which had been adjusted to pH 3.5 with glacial acetic acid (pH 3.5). The mixture was shaken by hand for 1 min and allowed to stand for 5 min, and then excess liquid was removed and replaced by the extract. The total volume was increased to 20 mL with glacial acetic acid, pH 3.5, and the mixture was shaken for 20 min. After centrifugation at 1180*g* for 15 min and recovery of the supernatant, the process was repeated and the supernatants were combined. The sample was dried down using a rotary evaporator and resuspended in 10 mL of 10 mM triethylammonium acetate (TEAA) at pH 7.

Further purification before fractionation by HPLC was achieved using a Sep-Pak C_{18} cartridge. The cartridge was wetted with 5 mL of 100% methanol, equilibrated with 10 mL of 10 mM TEAA, pH 7, loaded with the sample, and washed with a further 10 mL of 10 mM TEAA, pH 7, and the cytokinins were eluted in 10 mL of 50% methanol. The sample was reduced to less than 1 mL in volume on the rotary evaporator and stored in liquid nitrogen in a silanized 1.5-mL Eppendorf tube.

Fractionation of Cytokinins by HPLC

The equipment used to fractionate the cytokinins was as described in Turnbull and Hanke (1985). Separation of all 12 quantifiable cytokinins was achieved using a gradient of 15 to 37.5% (v/v) HPLC-grade methanol in 0.2 mM TEAA, pH 7, over 15 min, followed by continued elution with 37.5% methanol for a further 20 min, at a flow rate of 1.5 mL/min. A solution containing 0.5 nmol of each of the quantifiable cytokinins was run at the beginning and end of the day and after every two samples to establish the retention time on the column for each cytokinin. For each sample, 1.5-mL fractions were collected from the column in uncapped Eppendorf tubes. The fractions were dried down at 40°C under reduced pressure, resuspended in double-distilled water, and stored at −20°C until assayed.

Quantification of Cytokinins by Radioimmunoassay

Cytokinins in each HPLC fraction were assayed using the radioimmunoassay method described in Kraigher et al. (1991). The antibodies used had been raised in rabbits against zeatin riboside, dihydrozeatin riboside, and *iso*-pentenyl adenine riboside (C. Turnbull and A. Grayling, unpublished results). Cross-reactivity to each antibody was high within each family of cytokinins but relatively low between families (Grayling, 1990). All HPLC fractions were assayed with each of the three antibodies and the cytokinins present deduced from their HPLC retention times and cross-reactivities.

Radioimmunoassays were carried out in 1.5-mL Eppendorf tubes. Each tube contained 100 μL of phosphate-buffered saline including γ-globulin and the appropriate serum, 50 μL of tracer, and 50 μL of sample. Duplicate tubes were prepared for each sample. The final concentration of components in the assay mixture was as follows: 100 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0, 0.25 g/L NaN_3, 1 mg/mL γ-globulin, about 170 Bq of tracer (zeatin riboside-^{3}H-diol, dihydrozeatin riboside-^{3}H-diol, or *iso*-pentenyl adenine riboside-^{3}H-diol), and serum diluted so that about 50% of the tracer would be bound at equilibrium (1/2,000 for anti-zeatin riboside serum, 1/6,000 for anti-dihydrozeatin riboside serum, and 1/10,000 for anti-*iso*-pentenyl adenine riboside serum).

After mixing, the tubes were incubated at room temperature for 2 hr to allow antibody binding to reach equilibrium. Protein was precipitated by the addition of 200 μL of saturated ammonium sulfate and pelleted by centrifugation in an Eppendorf centrifuge model 5413 for 10 min. A sample of 200 μL of the supernatant was mixed with 2 mL of Hionic-Fluor scintillation fluid (Canberra Packard, Pangbourne, United Kingdom) and the amount of radioactivity quantified using a Tri-Carb 2000 scintillation counter (Canberra Packard). For each assay, standards containing 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 pmol of unlabeled cytokinin riboside in 50 μL of water were counted before the sample fractions and the results were used to draw a standard curve. This was done using the M-Spline curve-fitting procedure in the SecuRIA software package from Canberra Packard, which also calculated the amount of cytokinin in the fractions of each sample.

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